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# Orexin receptor type-1 antagonist SB-334867 decreases morphine-induced antinociceptive effect in formalin test

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ABSTRACT

Orexin-A and orexin-B are two neuropeptides selectively synthesized in the lateral hypothalamus (LH), a region involved in morphine induced analgesia and pain modulation. Furthermore, orexin-A has been reported to produce an analgesic effect in pain models, which was blocked by orexin-1 receptor antagonist SB-334867, but not naloxone. We studied the effects of intracerebroventricular (ICV) injection of SB-334867, a selective orexin receptor type-1 antagonist, on morphine-induced antinociceptive effect in formalin test in rats. Morphine injection at a dose of 1.5 mg/kg caused a significant decrease in the formalin-induced nociceptive behaviors in phase 1, interphase, and phase 2A, whereas at doses of 3, 6, and 10 mg/kg, a significant reduction in the formalin-induced nociceptive behaviors was observed in all phases. The ICV injection of SB-334867 alone had no effect on the formalin-induced nociceptive behaviors. Pre-treatment with SB-334867 at a dose of 0.5 nmol significantly attenuated the analgesia induced by morphine (at dose 1.5 mg/kg of morphine; interphase and phase 2B and at dose 3 mg/kg of morphine just phase 2B of formalin test). Also, pre-treatment with SB-334867 at a dose of 5 nmol considerably attenuated the morphine-induced analgesia (at dose 1.5 mg/kg of morphine; phase 1, interphase, and phase 2, at dose 3 and 6 mg/kg of morphine just phase 2 of formalin test). Pre-treatment with SB-334867 at a dose of 50 nmol remarkably attenuated the morphine-induced analgesia (at dose 1.5 and 3 mg/kg of morphine; in phase 1, interphase, and phase 2 and also at dose 6 mg/kg of morphine; phase 1 and phase 2B of formalin test). These data suggest that the antinociceptive effects of morphine in formalin test might be associated with orexin receptor type-1. Our findings reveal a new role for the lateral hypothalamus orexin neurons in the morphine-induced analgesia.



## 1. Introduction

Morphine, the most abundant alkaloid found in opium, is a potent analgesic and frequently used drug for the treatment of both acute and chronic pain. There are spinal and supra-spinal mechanisms governing the analgesic effect of systemically injected morphine (Barton et al., 1980). Morphine activates the top-down descending modulating inhibitory systems in the supraspinal regions such as the rostral ventromedial medulla (RVM), which finally inhibited the first synapse in the dorsal horn neurons (Basbaum and Fields, 1984). Also, through another mechanism, morphine directly affects the first synapse of dorsal horn neurons, inhibiting the transmission of nociceptive information to supra-spinal centers (Yaksh and Noueihed, 1985).

The lateral hypothalamus (LH) that exclusively expresses orexin has a very important role in the modulation of nociceptive behavior (Dafny et al., 1996; Franco and Prado, 1996; Holden and Pizzi, 2008). The stimulation of LH produces an antinociceptive effect in both acute and tonic pain (Dafny et al., 1996; Franco and Prado, 1996; Holden and Naleway, 2001). Additionally, the stimulation of LH elicits antinociception via relays to the brain stem sites such as; periaqueductal gray matter (PAG) and the RVM, which ultimately effect on descending inhibitory pathways (Behbehani et al., 1988). Morphine microinjection into the LH significantly decreases nociceptive behaviors on the tail flick and formalin-induced nociceptive behaviors (Fuchs and Melzack, 1995; Dafny et al., 1996; Franco and Prado, 1996), suggesting that the LH can be considered as one of the centers in the supraspinal region that modulates nociceptive behaviors (Lopez et al., 1991; Lopez and Cox, 1992; Fuchs and Melzack, 1995; Dafny et al., 1996; Franco and Prado, 1996). In a study by Jain et al., it was shown that the LH lesions produced a hyperalgesic effect as demonstrated by a decrease in the tail flick latency (Jain et al., 2001).

Recently, two new neuropeptides; the orexin-A and orexin-B (also called hypocretin-1 and hypocretin-2) have been described in the neurons of the LH and perifornical area. These neuropeptides are reported to have broad projections, leading to the recognition of their roles in

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the regulation of a variety of functions including feeding, sleep–wake cycle, cardiovascular function, hormone secretion (Ferguson and Samson, 2003; Siegel, 2003; Sakurai, 2005; Samson et al., 2005), and more recently the modulation of nociceptive processing (Bingham et al., 2001; Yamamoto et al., 2003a; Suyama et al., 2004; Mobarakeh et al., 2005b; Azhdari-Zarmehri et al., 2011; Sadeghi et al., 2013). The antinociceptive effect of orexin-A which is blocked by orexin-1 receptor antagonist SB-334867 but not naloxone, ruled out the involvement of endogenous opiate system in these effects (Bingham et al., 2001; Yamamoto et al., 2002; Cheng et al., 2003). Furthermore, the stress-induced antinociceptive effect was prevented by SB-334867 (Heidari-Oranjaghi et al., 2012) and the antagonism of orexin receptor 1 decreased the development of morphine tolerance and physical dependence (Erami et al., 2012b). In another study, we showed that the orexinergic projections from the LH to the ventral tegmental area and the nucleus accumbens are direct/indirectly involved in the antinociception induced by LH chemical stimulation, and orexin-1 and -2 receptors in the ventral tegmental area have a more substantial role in this phenomenon (Sadeghi et al., 2013; Azhdari-Zarmehri et al., 2013). Orexin-A and their receptors are localized in the areas of the brain and spinal cord associated with nociceptive processing such as the periaqueductal gray (PAG) and locus coeruleus (LC) (Peyron et al., 1998). These studies that emphasize the analgesic effect of orexin-A (Bingham et al., 2001; Chiou et al., 2010; Azhdari-Zarmehri et al., 2011; Ho et al., 2011; Erami et al., 2012a) and the pattern of localization of both peptide and receptor in the areas involved in pain modulation provide a strong evidence for orexin-A in having a role in the modulation of morphine-induced analgesia. Recently, using in-situ hybridization, Georgescu et al. (2003) have demonstrated that all orexin-A cells that respond to chronic morphine administration and opiate antagonist-precipitated morphine withdrawal also express the μ-opioid receptor, suggesting a direct mode of action (Georgescu et al., 2003).

The stimulation of LH or the microinjection of morphine into the LH produces an analgesic effect (Holden et al., 2002; Holden et al., 2005). Moreover, orexin-A is exclusively produced in the LH and since it has an essential role in pain modulation, and that the orexin induced antinociceptive effect mediated through orexin receptor 1, we hypothesized that the blockade of orexin receptor type-1 by SB-334867 prevents the morphine-induced antinociceptive effect in the formalin test. We used the formalin test as a tonic pain test to quantify the effect of the antagonism of orexin receptor type-1 on the morphine induced analgesia. The formalin-induced nociceptive behavior was used because it is a model of inflammation that lasts long enough, during which the experimental animals show a spontaneous response. Moreover, the pain stimulus is a continuous rather than a transient one and may thus have resemblance to some kinds of clinical pain and that the observations are made on animals that are restrained lightly or not at all.

## 2. Materials and methods

### 2.1. Subjects

All experiments involving the animals were conducted according to the policy of Iranian Convention for the Protection of Vertebrate Animals Used for the Experimental Purposes, and the protocol was approved by the Ethics Committee of the School of Medicine, Qazvin University of Medical Sciences, Qazvin, Iran. Adult Wistar rats (220–300 g) were purchased from Razi Institute (Karaj, Iran). Animals were housed in groups of 8 rats per big cage (cage size was 58 × 40 × 22 cm), at a temperature-controlled room, under a 12 h light–dark cycle with lights on at 7:00 to 19:00. Food and water were provided ad libitum. In all experiments, attention was paid to the regulations of the local authorities for handling of laboratory animals.

### 2.2. Drugs

Two percent formalin was diluted (Temad, Iran) with sterile physiological saline solution (Soha, Iran). Morphine sulfate (Temad, Iran) was dissolved in saline. OX1R antagonist; SB-334867 (N-(2-methyl-6-benzoxazolyl)-N′-1,5-naphthyridin-4-yl urea); (molecular weight = 356, Tocris), was dissolved in dimethyl sulfoxide (DMSO, stock solution) and diluted in saline on the day of experiment (DMSO was initially prepared as 10/100 in 0.9% w/v saline solution and further diluted 1/100 and 1/1000). All drugs were diluted in saline immediately before use on the morning of experiment.

### 2.3. Surgical preparation for ICV microinjections

To perform direct ICV administrations of drugs or respective vehicle, the guide cannula was implanted 7 days before the experiments. Rats were anesthetized with ketamine (100 mg/kg)/xylazine (10 mg/kg) and a 23-gauge stainless steel guide cannula, 2 mm long was stereotaxically (Stoelting) lowered until its tip was 2 mm above the right cerebroventricular by applying coordinates from the atlas of Paxinos and Watson (2005) [0.9 mm caudal to bregma, 1.8 mm lateral to the midline and 3.8 mm depth]. Using dental cement, the cannula was anchored on two stainless steel screws in the skull. Immediately after waking from surgery, rats were returned to their home cages to await the formalin test procedure. On the day of experiment, rats were transferred to individual cages and allowed to acclimatize for 60 min before they were put in an acrylic observation chamber. Direct ICV administration of drugs or respective vehicle, was conducted with a stainless steel cannula (30G, 0.3 mm outer diameter) connected by a polyethylene tube to a 26-gauge Hamilton syringe, inserted through the guide cannula and extended 2 mm beyond the tip of the guide cannula to reach the right cerebroventricular area. SB-334867 (0.5, 5 and 50 nmol; ICV) was microinjected 5 min (Heidari-Oranjaghi et al., 2012; Erami et al., 2012b) before morphine injection (1.5, 3, 6, and 10 mg/kg, S.C.) followed by the performance of formalin test 30 min later. Drug solutions or vehicles (5 μl) were injected ICV over a period of 60 s and the injection cannula was gently removed 1 min later. After 30 min, formalin was injected into the plantar surface of the right hind paw using a disposable insulin syringe with a fixed 30G needle.

### 2.4. Formalin test

Rats were moved to the test room at least 1 h before the commencement of experiment. Formalin tests were performed in clear plastic boxes (30 × 30 × 30 cm) with a mirror placed underneath at 45° angle to allow an unimpeded view of the animals' paws. Initially, rats were acclimatized for 30 min in an acrylic observation chamber followed by injecting 50 μl of 2% formalin subcutaneously into the plantar surface of the right hind paw using a 30 gauge needle. To ensure stable scores from the formalin-induced nociceptive behaviors, it was necessary to make sure that the needle was inserted through the skin and run for 5 mm under the skin. Each rat was immediately returned to the observation box and the behavioral recording began. Behavioral scoring was performed by a subtle modification of the method originally described by Dubuisson and Dennis (1977) in which they employed four behavioral categories. Nociceptive behaviors were scored as follows: 0, the injected paw was not favored; 1, the injected paw had little or no weight placed on it; 2, the injected paw was elevated and not in contact with any surface; and 3, the injected paw was licked or bit. Recording of nociceptive behaviors started immediately after formalin injection (time 0) and continued for 90 min. The scores of nociceptive behaviors for each 3-minute interval were calculated as the weighted average of the number of seconds, spent in each nociceptive behavior, from the beginning of experiment. The scores were recorded in vehicle rats as well as those receiving the drug. In each group, the average behavioral responses of each rat during the first (1–7 min), inter-phase (8–14),

phase 2A (15–60), and phase 2B (61–90) were separately evaluated and the data were reported as the time average of nociceptive behaviors.

### 2.5. Experimental protocols

The formalin test was the experimental procedure used in our study to examine the effect of antagonizing OX1R on the morphine antinociceptive effect. Three sets of experiments were considered in the formalin test: (Experiment 1) Rats were given only morphine injection (1.5, 3, 6 and 10 mg/kg) followed by the performance of formalin test. (Experiment 2) Rats had ICV microinjection of SB-334867 (0.5, 5 and 50 nmol) or 1% DMSO followed by morphine injection (1.5 mg/kg) and formalin test was pursued 30 min later. (Experiment 3) Rats were given SB-334867 (0.5, 5 and 50 nmol) by ICV microinjection or 1% DMSO followed by morphine injection (3 mg/kg) and 30 min later, the formalin test was performed. (Experiment 4) Rats received SB-334867 (0.5, 5 and 50 nmol) by ICV microinjection or 1% DMSO followed by morphine injection (6 mg/kg) and after 30 min, the formalin test was carried out. In all experiments, morphine was microinjected subcutaneously; SB-334867 microinjected into the right cerebroventricular area, and formalin injected into the plantar surface of the right hind paw.

### 2.6. Data analysis

Data were presented as mean ± SEM and analyzed by one-way analysis of variance between the groups. The total time points of 90 minutes duration were divided into four phases as phase 1 (minutes 0 to 7), interphase (minutes 8 to 14), phase 2A (minutes 15 to 60), and phase 2B (minutes 61 to 90). The Tukey's test was employed to determine where a significant difference occurred. The defined level for statistical significance was $P < 0.05$.

## 3. Results

### 3.1. Effects of different doses of morphine through subcutaneous injection on formalin-induced nociceptive behaviors

Fig. 1 indicates the results of experiment 1. Formalin produced typical biphasic pain responses. The first and second phases were separated by a brief inter-phase where little or no nociceptive behavior was observed in the control group (Fig. 1). Morphine injection at 1.5 mg/kg 30 min before the formalin test significantly decreased formalin-induced nociceptive behaviors in phase 1 ($p < 0.05$), interphase ($p < 0.001$), and phase 2A ($p < 0.05$) compared with the control group (Fig. 1); morphine injection at 3 and 6 mg/kg 30 min before the formalin test significantly reduced formalin-induced nociceptive behaviors in phase 1 ($p < 0.05$), interphase (for morphine at 3 mg/kg, $p < 0.01$; and for morphine at 6 mg/kg, $p < 0.001$), and phases 2A and 2B ($p < 0.001$) compared with the control group (Fig. 1). Moreover, the injection of 10 mg/kg morphine completely suppressed formalin-induced nociceptive behaviors in both phases 1 and 2 ($p < 0.001$).

### 3.2. Effects of different doses of SB-334867 (ICV) on morphine-induced analgesia in formalin test

Fig. 2 demonstrates the results of experiment 2. Morphine injection at 1.5 mg/kg before the formalin test, significantly decreased formalin-induced nociceptive behaviors in phase 1 ($p < 0.05$), interphase ($p < 0.001$), and phase 2A ($p < 0.05$) compared with the control group (Fig. 2). Pre-treatment with orexin receptor type-1 antagonist, SB-334867 at a dose of 0.5 nmol significantly attenuated morphine-induced analgesia in interphase ($p < 0.05$, Fig. 2), and phase 2B compared with the control group ($p < 0.05$, Fig. 2). Pre-treatment with SB-334867 at a dose of 5 nmol, significantly attenuated morphine-induced analgesia in interphase ($p < 0.05$) and when used at a higher dose (50 nmol), morphine-induced analgesia was considerably attenuated in phase 1 ($p < 0.05$), interphase ($p < 0.01$), and the first and second parts of phase 2 compared with the control group ($p < 0.01$, Fig. 2).

Fig. 3 shows the results of experiment 3. The injection of 3 mg/kg of morphine alone produced antinociceptive behaviors in the formalin test as demonstrated by a substantial decrease in the nociceptive behavior in phase 1 ($p < 0.05$), interphase ($p < 0.01$), and the first and second parts of phase 2 compared with the control group ($p < 0.001$, Fig. 3). Also, pre-treatment with SB-334867 at a dose of 0.5 nmol remarkably attenuated morphine-induced analgesia in phase 2B when compared with the control group ($p < 0.05$). In addition, the administration of SB-334867 at a dose of 5 nmol significantly attenuated morphine-induced analgesia in phase 1 ($p < 0.05$), interphase ($p < 0.05$), and the first ($p < 0.05$) and the second parts of phase2 ($p < 0.01$). Furthermore, pre-treatment with SB-334867 at a dose of 50 nmol notably attenuated morphine-induced analgesia in phase 1 ($p < 0.001$), interphase ($p < 0.05$), and the first and second parts of phase 2 when compared to the control group ($p < 0.01$, Fig. 3).

Finally, Fig. 4 illustrates the results of experiment 4 in which the injection of 6 mg/kg of morphine alone produced antinociceptive behaviors in the formalin test as revealed by a considerable decrease in nociceptive behavior in phase1 ($p < 0.001$), interphase ($p < 0.001$), and the first and second parts of phase 2 compared with the control group

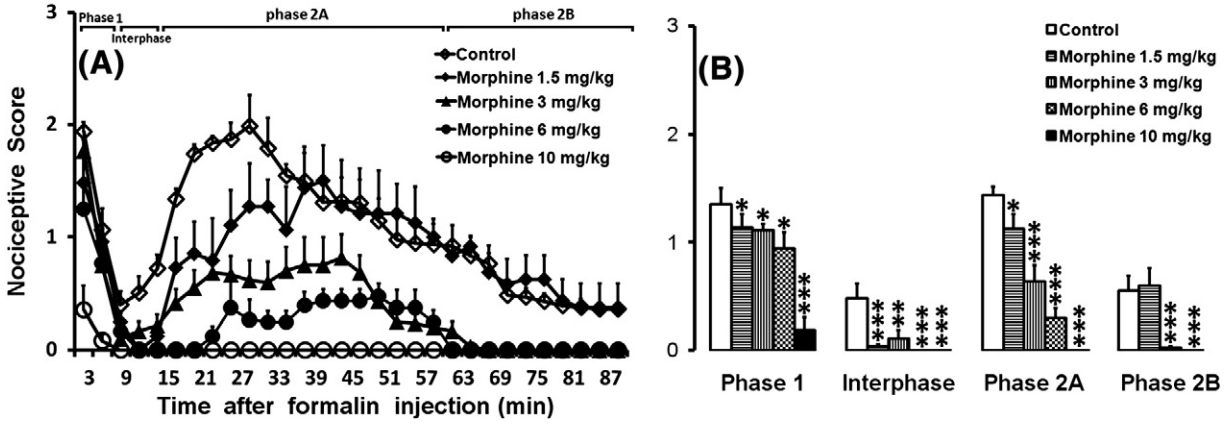


**Fig. 1.** Formalin-induced nociceptive behaviors (mean ± SEM of 6 rats per group) following subcutaneous injection of morphine (1.5. 3, 6, and 10 mg/kg) measured every 3 min for 90 min (A) and the bar chart (B). The columns represent the average nociceptive scores in each phase: phase 1 (minutes 1–7), inter-phase (minutes 8–14), phase 2A (minutes 15–60), and phase 2 (minutes 61–90). Rats received morphine 30 min before formalin injection. Recording of nociceptive behaviors began immediately after formalin injection (time 0) and continued for 90 min. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ compared with control group.

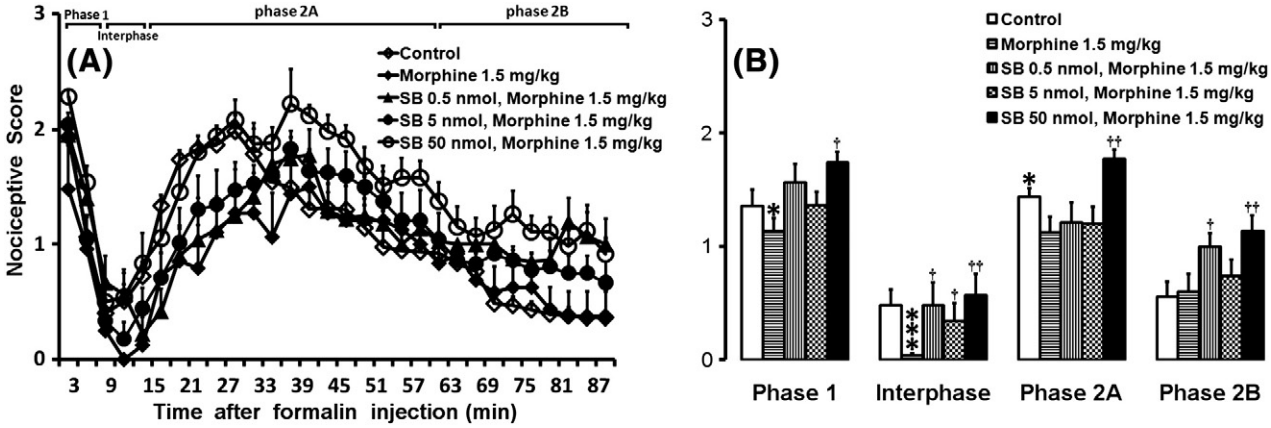


**Fig. 2.** Formalin-induced nociceptive behaviors (mean ± SEM of 6–8 rats per group) following ICV administration of SB-334867 (0.5, 5 and 50 nmol) + morphine 1.5 mg/kg, measured every 3 min for 90 min (A), the bar chart for each phase (B). In B section, the columns represent the average nociceptive scores in each phase: phase 1 (minutes 1–7), inter-phase (minutes 8–14), phase 2A (minutes 15–60) and phase 2 (minutes 61–90). Rats received morphine 30 min before formalin injection. Recording of nociceptive behaviors began immediately after formalin injection (time 0) and continued for 90 min. *P < 0.05; **P < 0.01 and ***P < 0.001 compared with control group. †P < 0.05; ††P < 0.01 and †††P < 0.001 compared with morphine induced analgesia group.

($p < 0.001$, Fig. 4). Moreover, pre-treatment with SB-334867 at a dose of 0.5 nmol produced no significant attenuation of morphine-induced antinociceptive effect in the formalin test ($p > 0.05$, Fig. 4). Also, pre-treatment with SB-334867 at a dose of 5 nmol considerably attenuated morphine-induced analgesia at the first ($p < 0.05$) and second parts of phase 2 ($p < 0.01$). Furthermore, SB-334867 at a dose of 50 nmol significantly attenuated morphine-induced analgesia in phase 1 ($p < 0.001$) and phase 2B when compared to the control group ($p < 0.001$, Fig. 4).

To investigate the effect of different doses of orexin receptor type-1 on the antinociceptive effect of different doses of morphine, we analyzed our data for phase 1 (min 0–7) and phase 2 (min 15–90) and the results were included in Table 1 in which the reversal percentages of morphine-induced antinociception by different doses of SB-334867 are shown. As demonstrated in Table 1, the ICV administration of SB-334867 at 0.5 nmol failed to produce any effect on antinociceptive responses of morphine (1.5, 3 and 6 mg/kg) in phase 1 [$F(2, 16) = 0.552$, $P = 0.590$], and phase 2 [$F(2, 16) = 3.852$, $P = 0.079$], revealed by one-way ANOVA and the Newman–Keuls multiple comparison tests. The ICV administration of SB-334867 at 5 nmol failed to produce any effect on the antinociceptive responses of morphine (1.5, 3 and 6 mg/kg) in phase 1 [$F(2, 17) = 1.466$, $P = 0.281$] but showed a significant effect on phase 2 [$F(2, 17) = 6.873$, $P = 0.052$], demonstrated by one-way ANOVA and the Newman–Keuls multiple comparison tests. One-way ANOVA followed by the Newman–Keuls multiple comparison tests showed that the antinociceptive response of morphine (1.5, 3 and 6 mg/kg) was suppressed by the injection of SB-334867 at 50 nmol concentration in phase 1 [$F(2, 15) = 15.489$, $P = 0.000$] and phase 2 [$F(2, 15) = 12.313$, $P < 0.001$] (Table 1.).

The injection of 10 mg/kg morphine completely suppressed formalin-induced nociceptive behaviors in both phases 1 and 2 ($p < 0.001$, Fig. 1). Pre-treatment with SB-334867 at doses of 0.5, 5, and 50 nmol produced no significant attenuation of morphine-induced antinociceptive effect at 10 mg/kg in the formalin test ($p > 0.05$, data not shown).

## 4. Discussion

It is well-known that morphine induced analgesia is mediated through (direct and indirect) spinal and supraspinal sites (Yaksh and Noueihed, 1985; Dafny et al., 1996; Franco and Prado, 1996). Investigations reported from several laboratories support the involvement of orexin-A in the modulation of nociceptive behavior (Yamamoto et al., 2003b; Holland et al., 2005; Mobarakeh et al., 2005a,b; Chiou et al.,

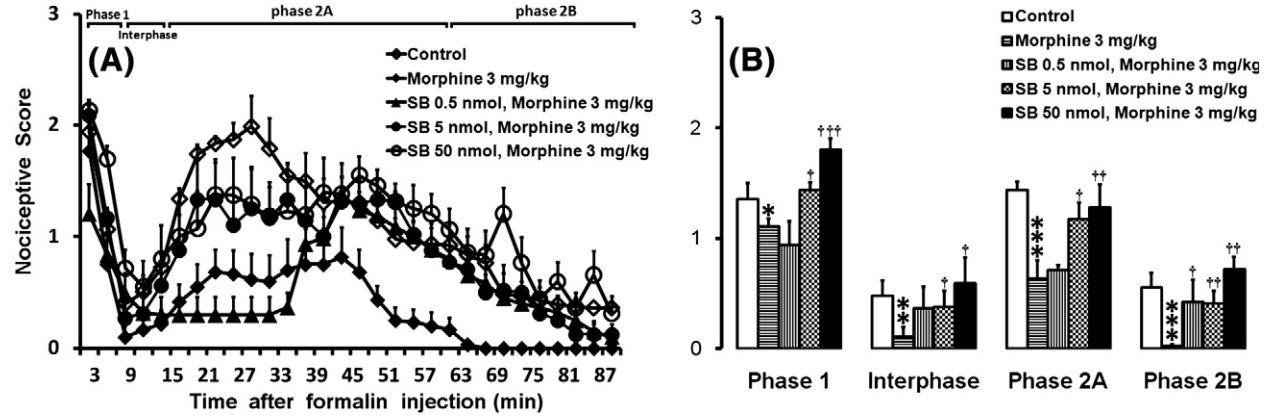


**Fig. 3.** Formalin-induced nociceptive behaviors (mean ± SEM of 6–8 rats per group) following ICV administration of SB-334867 (0.5, 5 and 50 nmol) + morphine 3 mg/kg, measured every 3 min for 90 min (A), the bar chart for each phase (B). In B section, the columns represent the average nociceptive scores in each phase: phase 1 (minutes 1–7), inter-phase (minutes 8–14), phase 2A (minutes 15–60) and phase 2 (minutes 61–90). Rats received morphine 30 min before formalin injection. Recording of nociceptive behaviors began immediately after formalin injection (time 0) and continued for 90 min. *P < 0.05; **P < 0.01 and ***P < 0.001 compared with control group. †P < 0.05; ††P < 0.01 and †††P < 0.001 compared with morphine induced analgesia group.

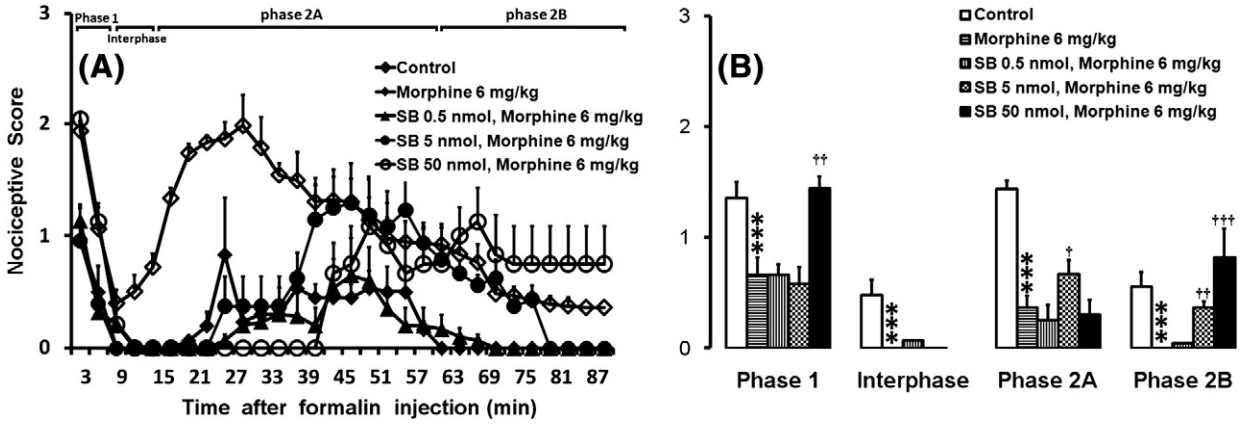


**Fig. 4.** Formalin-induced nociceptive behaviors (mean ± SEM of 6–8 rats per group) following ICV administration of SB-334867 (0.5, 5 and 50 nmol) + morphine 6 mg/kg, measured every 3 min for 90 min (A), the bar chart for each phase (B). In B section, the columns represent the average nociceptive scores in each phase: phase 1 (minutes 1–7), inter-phase (minutes 8–14), phase 2A (minutes 15–60) and phase 2 (minutes 61–90). Rats received morphine 30 min before formalin injection. Recording of nociceptive behaviors began immediately after formalin injection (time 0) and continued for 90 min. *P < 0.05; **P < 0.01 and ***P < 0.001 compared with control group. †P < 0.05; ††P < 0.01 and †††P < 0.001 compared with morphine induced analgesia group.

2010; Azhdari-Zarmehri et al., 2011; Erami et al., 2012a; Sadeghi et al., 2013). The present investigation was designed to determine the possible involvement of orexin in morphine induced antinociception. In the current study, the subcutaneous administration of morphine (1.5, 3, 6, 10 mg/kg S.C.) produced clear dose-related antinociceptive effects as evaluated by the formalin test. The antinociceptive effects of morphine administration were decreased by a selective orexin receptor type-1 antagonist, SB-334867 (0.5, 5 and 50 nmol; ICV) but not in a dose dependent manner. These results suggest that some parts of the analgesic effect of morphine are mediated by orexinergic system through the activation of orexin receptor type-1. The involvement of the orexinergic system which mediates the morphine antinociception, as demonstrated in the present investigation, is well supported by previous studies. It has been reported that orexin-A reduces the level of thermal hyperalgesia in the mouse carrageenan test and produces an analgesic effect in the mouse hot plate test. These effects of orexin-A which are mediated by the activation of orexin receptor type-1, as SB-334867 and not naloxone, prevent orexin-A induced analgesia (Bingham et al., 2001). Yamamoto et al. in 2002 and 2003 showed that the application of orexin-A produced an anti-mechanical allodynic effect and decreased both the thermal hyperalgesia induced by the paw carrageen injection and the formalin-induced nociceptive behaviors. Naloxone pre-treatment produced no effect on the antinociceptive effect of orexin-A in the formalin test in rats (Yamamoto et al., 2002, 2003a). In another study, they also showed that orexin-A has an anti-mechanical allodynia induced by the partial sciatic nerve ligation (a model of neuropathic pain) in rats (Yamamoto et al., 2003a). There are several documents that demonstrate that both the spinal and supraspinal mechanisms contribute to the antinociceptive effects of orexin-A (Bingham et al., 2001; Yamamoto et al., 2002, 2003a; Azhdari-Zarmehri et al., 2011; Erami et al., 2012a). This is consistent with the localization of orexin receptor type-1 in the nociceptive area of the spinal cord and the brain (Peyron et al., 1998; Trivedi et al., 1998; Hervieu et al., 2001). A previous study in our lab showed that stress-induced antinociceptive effect is prevented by SB-334867 (Heidari-Oranjaghi et al., 2012) and the antagonism of orexin receptor type-1decreased the development of morphine tolerance and dependence (Erami et al., 2012b).

In the present study, our results showed that pre-treatment with SB-334867 (0.5, 5 and 50 nmol) reversed the analgesic effect of morphine in a dose dependent manner, however, several previous studies have shown that naloxone pre-treatment had no effect on the analgesic effect of orexin-A induced analgesia (Bingham et al., 2001; Yamamoto et al., 2002; Cheng et al., 2003). This led us to suggest that the analgesic effect of orexin-A was unlikely to be mediated by endogenous opioid system at the supra-spinal and spinal cord levels and instead, the antinociceptive effect of morphine in the formalin test may have been caused by the modulation of orexin-A pathway in the brain or/and spinal cord. This is consistent with the facts that: 1) Electrical or chemical stimulations of LH were found to produce an analgesic effect (Fuchs and Melzack, 1995; Dafny et al., 1996; Holden and Pizzi, 2008). 2) the microinjection of morphine into LH also produced an analgesic effect (Fuchs and Melzack, 1995; Dafny et al., 1996; Holden and Pizzi, 2008). 3) orexin-A has an important role in pain modulation and is reported to produce an analgesic effect (Chiou et al., 2010; Azhdari-Zarmehri et al., 2011; Erami et al., 2012a). 4) Previous studies with in-situ hybridization have indicated that the μ-opioid receptor is expressed in the LH region; Georgescu et al. (2003) used immunofluorescence with double-labeling to examine the presence of μ-opioid receptors on orexin cells in the LH (Georgescu et al., 2003).

The excitatory effect of morphine on orexin neurons of LH might not be due to the direct effect of morphine on excitatory synapse transmission, but may result from the inhibition on interneurons, as disinhibition mechanism that has been described in other regions of brain (Fields et al., 1983; Zhu et al., 2008). For example; activation of mu opioid receptor effect on inhibitory recurrent circuits in the dentate gyrus and therewith, indirectly plays a regulatory role for excitatory neurotransmission

**Table 1**
Reversal percentages of morphine induced antinociception by different doses of SB-334867 in the formalin test. * P < 0.05 and ** P < 0.01 for the significance of asterisk in the below table.

| SB-334867 | SB-334867 0.5 nmol | | SB-334867 5 nmol | | SB-334867 50 nmol | |
|---|---|---|---|---|---|---|
| Morphine | Phase 1 | Phase 2 | Phase 1 | Phase 2 | Phase 1 | Phase 2 |
| Mor 1.5 | 0.3 ± 11.1 | 37.1 ± 16.1 | −12.6 ± 10.3 | 14.9 ± 24.5 | 12.0 ± 7.8 | 73.9 ± 19.8 |
| Mor 3 | −0.6 ± 22.5 | 943.7 ± 296.2* | 24.3 ± 8.1* | 869.0 ± 203.1* | 50.8 ± 12.4* | 1577.6 ± 217.6* |
| Mor 6 | −7.5 ± 16.1 | −10.8 ± 43.4 | 7.1 ± 8.2* | 81.1 ± 46.2 | 90.2 ± 20.2** | 13.9 ± 44.5 |

through GABAergic disinhibition (Akaishi et al., 2000). Furthermore, synaptic disinhibition is one possible mechanism by which morphine increases neurotransmitter release in the brain nuclei (Zhu et al., 2008).

Orexin-induced analgesia involves both spinal and supraspinal mechanisms. The dens projections of orexin cells to the marginal zone, lamina 1, and the entire segments of the spinal cord proposed that orexin may be implicated in pain processing and thermal sensation (van den Pol, 1999). In some sections of Van Den Pol's study, some cells of the marginal zone were restricted by the orexin-immunoreactive boutons, suggesting that orexin fibers selectively innervate subpopulations of cells in the marginal zone which might either be associated with a spatial sensation, pain information, or modulating specific ascending fibers (Cedarbaum and Aghajanian, 1978; Narotzky and Kerr, 1978). Supraspinal analgesia involves effects of orexin-A at the level of the PAG. The intra-PAG administration of orexin A produced an antinociceptive effect which was blocked by SB 334867 (Azhdari-Zarmehri et al., 2011; Ho et al., 2011) or AM 251 (Ho et al., 2011). In the slices of the ventrolateral PAG, orexin-A, through the receptor 1, suppressed the GABAergic induced inhibitory postsynaptic current. The postsynaptic OX1 receptor activation in the ventrolateral PAG can engage a Gq-protein coupled PLCβ-DAGLα enzymatic pathway to produce 2-AG, leading to a disinhibition phenomenon followed by activating the top-down descending pain inhibitory pathway, and these in turn explain the analgesic effect of orexin-A in the ventrolateral PAG (Ho et al., 2011).

Our result also showed that the ICV injection of SB-334867 alone had no effect on the formalin test at the doses usually used to antagonize the analgesic effect of orexin-A in the formalin test. In this respect, our results are consistent with a previous study that showed that the intrathecal injection of SB-334867 alone had no effect in both formalin and hot plate tests (Yamamoto et al., 2002). However, our findings are incompatible with those of Bingham et al. (2001) in which it was demonstrated that the intraperitoneal injection of SB-334867 produced a pro-hyperalgesic effect in the mouse carrageenan-induced thermal hyperalgesia test (Bingham et al., 2001). Based on a report by Yamamoto et al. in 2002, these data might suggest that a tonic orexin-A inhibitory system does not exist in the central nervous system during the formalin test (Yamamoto et al., 2002) however, there may be a tonically activated orexin-A inhibitory system present during a carrageenan-induced thermal hyperalgesia test in mice (Bingham et al., 2001). To justify this difference, there are several possibilities: Although both subcutaneous formalin injection and carrageenan injection induce a localized inflammation, nevertheless the formalin injection of the paw induces a nociceptive behavior for about 1 h (Wheeler-Aceto et al., 1990; Yamamoto and Yaksh, 1992) whereas the edema induced by the paw carrageenan injection lasts longer than the formalin test and in addition, the thermal hyperalgesia in the carrageenan test occurs about 2 h after the injection (Wheeler-Aceto et al., 1990; Yamamoto and Yaksh, 1992; Yamamoto et al., 2002). This may reflect the strain differences, different routes of administration, differences between the models used (carrageenan hyperalgesia and formalin tests), and methodological differences (e.g. lighting, noise, odors, handling stress or anesthesia prior to formalin injection and might differences in data collection times between laboratories; all known to influence the test).

In conclusion, based on the findings of both the previous and the present studies, we would like to suggest that the orexinergic system might provide a possible pathway for morphine induced analgesia, promising a new potential therapeutic target in the treatment of pain. However, further in vitro and in vivo studies are necessary to clarify how the orexinergic system is involved in morphine pain modulation.

## Conflict of interest

There is no financial or other conflict of interest associated with the present study.

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## References

Akaishi T, Saito H, Ito Y, Ishige K, Ikegaya Y. Morphine augments excitatory synaptic transmission in the dentate gyrus through GABAergic disinhibition. Neurosci Res 2000;38:357–63.
Azhdari-Zarmehri H, Reisi Z, Vaziri A, Haghparast A, Shaigani P, Haghparast A. Involvement of orexin-2 receptors in the ventral tegmental area and nucleus accumbens in the antinociception induced by the lateral hypothalamus stimulation in rats. Peptides 2013;47:94–8.
Azhdari-Zarmehri H, Semnanian S, Fathollahi Y, Erami E, Khakpay R, Azizi H, et al. Intra-periaqueductal gray matter microinjection of orexin-A decreases formalin-induced nociceptive behaviors in adult male rats. J Pain 2011;12:280–7.
Barton C, Basbaum AI, Fields HL. Dissociation of supraspinal and spinal actions of morphine: a quantitative evaluation. Brain Res 1980;188:487–98.
Basbaum AI, Fields HL. Endogenous pain control systems: brainstem spinal pathways and endorphin circuitry. Annu Rev Neurosci 1984;7:309–38.
Behbehani MM, Park MR, Clement ME. Interactions between the lateral hypothalamus and the periaqueductal gray. J Neurosci 1988;8:2780–7.
Bingham S, Davey PT, Babbs AJ, Irving EA, Sammons MJ, Wyles M, et al. Orexin-A, an hypothalamic peptide with analgesic properties. Pain 2001;92:81–90.
Cedarbaum JM, Aghajanian GK. Afferent projections to the rat locus coeruleus as determined by a retrograde tracing technique. J Comp Neurol 1978;178:1–16.
Cheng JK, Chou RC, Hwang LL, Chiou LC. Antiallodynic effects of intrathecal orexins in a rat model of postoperative pain. J Pharmacol Exp Ther 2003;307:1065–71.
Chiou LC, Lee HJ, Ho YC, Chen SP, Liao YY, Ma CH, et al. Orexins/hypocretins: pain regulation and cellular actions. Curr Pharm Des 2010;16:3089–100.
Dafny N, Dong WQ, Prieto-Gomez C, Reyes-Vazquez C, Stanford J, Qiao JT. Lateral hypothalamus: site involved in pain modulation. Neuroscience 1996;70:449–60.
Dubuisson D, Dennis SG. The formalin test: a quantitative study of the analgesic effects of morphine, meperidine, and brain stem stimulation in rats and cats. Pain 1977;4:161–74.
Erami E, Azhdari-Zarmehri H, Ghasemi-Dashkhasan E, Esmaeili MH, Semnanian S. Intra-paragigantocellularis lateralis injection of orexin-A has an antinociceptive effect on hot plate and formalin tests in rat. Brain Res 2012a;1478:16–23.
Erami E, Azhdari-Zarmehri H, Rahmani A, Ghasemi-Dashkhasan E, Semnanian S, Haghparast A. Blockade of orexin receptor 1 attenuates the development of morphine tolerance and physical dependence in rats. Pharmacol Biochem Behav 2012b;103:212–9.
Ferguson AV, Samson WK. The orexin/hypocretin system: a critical regulator of neuroendocrine and autonomic function. Front Neuroendocrinol 2003;24:141–50.
Fields HL, Vanegas H, Hentall ID, Zorman G. Evidence that disinhibition of brain stem neurones contributes to morphine analgesia. Nature 1983;306:684–6.
Franco AC, Prado WA. Antinociceptive effects of stimulation of discrete sites in the rat hypothalamus: evidence for the participation of the lateral hypothalamus area in descending pain suppression mechanisms. Braz J Med Biol Res 1996;29:1531–41.
Fuchs PN, Melzack R. Analgesia induced by morphine microinjection into the lateral hypothalamus of the rat. Exp Neurol 1995;134:277–80.
Georgescu D, Zachariou V, Barrot M, Mieda M, Willie JT, Eisch AJ, et al. Involvement of the lateral hypothalamic peptide orexin in morphine dependence and withdrawal. J Neurosci 2003;23:3106–11.
Heidari-Oranjaghi N, Azhdari-Zarmehri H, Erami E, Haghparast A. Antagonism of orexin-1 receptors attenuates swim- and restraint stress-induced antinociceptive behaviors in formalin test. Pharmacol Biochem Behav 2012;103:299–307.
Hervieu GJ, Cluderay JE, Harrison DC, Roberts JC, Leslie RA. Gene expression and protein distribution of the orexin-1 receptor in the rat brain and spinal cord. Neuroscience 2001;103:777–97.
Ho YC, Lee HJ, Tung LW, Liao YY, Fu SY, Teng SF, et al. Activation of orexin 1 receptors in the periaqueductal gray of male rats leads to antinociception via retrograde endocannabinoid (2-arachidonoylglycerol)-induced disinhibition. J Neurosci 2011;31:14600–10.
Holden JE, Naleway E. Microinjection of carbachol in the lateral hypothalamus produces opposing actions on nociception mediated by alpha(1)- and alpha(2)-adrenoceptors. Brain Res 2001;911:27–36.
Holden JE, Pizzi JA. Lateral hypothalamic-induced antinociception may be mediated by a substance P connection with the rostral ventromedial medulla. Brain Res 2008;1214:40–9.
Holden JE, Van Poppel AY, Thomas S. Antinociception from lateral hypothalamic stimulation may be mediated by NK(1) receptors in the A7 catecholamine cell group in rat. Brain Res 2002;953:195–204.
Holden JE, Farah EN, Jeong Y. Stimulation of the lateral hypothalamus produces antinociception mediated by 5-HT1A, 5-HT1B and 5-HT3 receptors in the rat spinal cord dorsal horn. Neuroscience 2005;135:1255–68.

Holland PR, Akerman S, Goadsby PJ. Orexin 1 receptor activation attenuates neurogenic dural vasodilation in an animal model of trigeminovascular nociception. J Pharmacol Exp Ther 2005;315:1380–5.
Jain JS, Mathur R, Sharma R, Nayar U. Reversal of hyperalgesia by transplantation in lateral hypothalamic lesioned rats. Neurobiology (Bp) 2001;9:17–22.
Lopez R, Cox VC. Analgesia for tonic pain by self-administered lateral hypothalamic stimulation. Neuroreport 1992;3:311–4.
Lopez R, Young SL, Cox VC. Analgesia for formalin-induced pain by lateral hypothalamic stimulation. Brain Res 1991;563:1–6.
Mobarakeh JI, Takahashi K, Sakurada S, Nishino S, Watanabe H, Kato M, et al. Enhanced antinociception by intracerebroventricularly administered orexin A in histamine H1 or H2 receptor gene knockout mice. Pain 2005a;118:254–62.
Mobarakeh JI, Takahashi K, Sakurada S, Nishino S, Watanabe H, Kato M, et al. Enhanced antinociception by intracerebroventricularly and intrathecally-administered orexin A and B (hypocretin-1 and -2) in mice. Peptides 2005b;26:767–77.
Narotzky RA, Kerr FW. Marginal neurons of the spinal cord: types, afferent synaptology and functional considerations. Brain Res 1978;139:1–20.
Paxinos G, Watson C. The rat brain in stereotaxic coordinates. New York: Academic Press; 2005.
Peyron C, Tighe DK, van den Pol AN, de LL, Heller HC, Sutcliffe JG, et al. Neurons containing hypocretin (orexin) project to multiple neuronal systems. J Neurosci 1998;18:9996–10015.
Sadeghi S, Raeisi Z, zhdari-Zarmehri H, Haghparast A. Involvement of orexin-1 receptors in the ventral tegmental area and the nucleus accumbens in antinociception induced by lateral hypothalamus stimulation in rats. Pharmacol Biochem Behav 2013;10.
Sakurai T. Roles of orexin/hypocretin in regulation of sleep/wakefulness and energy homeostasis. Sleep Med Rev 2005;9:231–41.
Samson WK, Taylor MM, Ferguson AV. Non-sleep effects of hypocretin/orexin. Sleep Med Rev 2005;9:243–52.
Siegel JM. The narcoleptic borderland. Sleep Med 2003;4:3–4.
Suyama H, Kawamoto M, Shiraishi S, Gaus S, Kajiyama S, Yuge O. Analgesic effect of intrathecal administration of orexin on neuropathic pain in rats. In Vivo 2004;18:119–23.
Trivedi P, Yu H, MacNeil DJ, Van der Ploeg LH, Guan XM. Distribution of orexin receptor mRNA in the rat brain. FEBS Lett 1998;438:71–5.
van den Pol AN. Hypothalamic hypocretin (orexin): robust innervation of the spinal cord. J Neurosci 1999;19:3171–82.
Wheeler-Aceto H, Porreca F, Cowan A. The rat paw formalin test: comparison of noxious agents. Pain 1990;40:229–38.
Yaksh TL, Noueihed R. The physiology and pharmacology of spinal opiates. Annu Rev Pharmacol Toxicol 1985;25:433–62.
Yamamoto T, Yaksh TL. Comparison of the antinociceptive effects of pre- and posttreatment with intrathecal morphine and MK801, an NMDA antagonist, on the formalin test in the rat. Anesthesiology 1992;77:757–63.
Yamamoto T, Nozaki-Taguchi N, Chiba T. Analgesic effect of intrathecally administered orexin-A in the rat formalin test and in the rat hot plate test. Br J Pharmacol 2002;137:170–6.
Yamamoto T, Saito O, Shono K, Aoe T, Chiba T. Anti-mechanical allodynic effect of intrathecal and intracerebroventricular injection of orexin-A in the rat neuropathic pain model. Neurosci Lett 2003a;347(3):183–6. [8-28, Ref Type: Patent].
Yamamoto T, Saito O, Shono K, Hirasawa S. Activation of spinal orexin-1 receptor produces anti-allodynic effect in the rat carrageenan test. Eur J Pharmacol 2003b;481:175–80.
Zhu Z, Bowman HR, Baghdoyan HA, Lydic R. Morphine increases acetylcholine release in the trigeminal nuclear complex. Sleep 2008;31:1629–37.